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(54) Title: INHIBITORS OF ALTERNATIVE ALLELES OF GENES AS A BASIS FOR CANCER THERAPEUTIC AGENTS

## (57) Abstract

This invention is directed to a therapeutic strategy, involving (1) identification of alternative alleles of genes coding for proteins vital for cell viability or cell growth and the loss of one of these alleles in cancer cells due to loss of heterozygosity (LOH) and (2) the development of inhibitors with high specificity for the single remaining alternative allele of the vital gene retained by the cancer cell after LOH. The inhibitors of this invention are specific for one alternative allele of a gene that codes for a protein vital to cell viability or cell growth. The targeted gene has two alternative alleles in which the inhibitors of this invention blocks only one of the two alternative alleles still present in the cancer cells. Exposure to the inhibitor inhibits or kills cancer cells which have undergone LOH. Protein is still capable of being expressed in the normal cells exposed to the inhibitor by the unblocked alternative allele. This differential effect of the inhibitor on cancer cells and normal cells accounts for the high therapeutic index of the inhibitors of this invention when used as antineoplastic agents.

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# INHIBITORS OF ALTERNATIVE ALLELES OF GENES AS A BASIS FOR CANCER THERAPEUTIC AGENTS

5

## Field of the Invention

This application is directed to therapeutic inhibitors to treat cancer and  
10 strategies for the development of cancer treatments having a high therapeutic  
index.

## BACKGROUND OF THE INVENTION

The targeting of chemotherapeutic agents to tumor cells is one of the  
15 most heavily investigated areas in biomedical research today. Although  
effective antineoplastic agents have been and continue to be discovered, there  
remains the immense problem of targeting these highly toxic agents  
specifically to tumor cells so that they do not also kill normal somatic cells  
and thereby cause permanent damage to vital organs or even death. Indeed,  
20 one measure of the clinical usefulness of any antineoplastic agent is its  
therapeutic index: the ratio of the median lethal dose (LD<sub>50</sub>) to the median  
effective dose (ED<sub>50</sub>) of the drug. With some cancer therapeutics this ratio is  
close to one, indicating a high level of toxic side effects to the patient.

25

## SUMMARY OF THE INVENTION

Cancer cells almost invariably undergo a loss of genetic material (DNA)  
when compared to normal cells. This deletion of genetic material is  
technically referred to as "loss of heterozygosity" (LOH). Recognizing that  
almost all, if not all, varieties of cancer undergo LOH, and that these regions  
30 are quite extensive, the inventor evaluated the genetic content of deleted  
regions in cancer cells and realized that genes vital for cell viability or cell

growth are frequently deleted. Further investigation has culminated in the discovery that the loss of genetic material from cancer cells sometimes results in the selective loss of one of two alleles of a certain vital gene at a particular locus on the chromosome. Based on this analysis, the inventor has devised a  
5 unique therapeutic strategy for the treatment of cancer. This strategy will result in agents characterized by a high therapeutic index. The strategy includes (1) identification of alternative alleles of genes coding for proteins vital for cell viability or growth; (2) identification of the absence of one of these alleles in cancer cells and (3) development of specific inhibitors of the  
10 single remaining allele of the vital gene retained by the cancer cell after LOH.

The inhibitors described in this invention are specific for one allele of a gene that codes for a protein vital to cell growth or cell viability. Exposure to the inhibitor inhibits proliferation or kills cancer cells which have undergone genetic deletion such that the cells only express the single allelic form of the  
15 vital protein against which the inhibitor is targeted. Normal cells exposed to the inhibitor are spared from the toxic effects of the inhibitor because the remaining activity of the unblocked alternative allele is adequate to permit continued cell viability and growth. This differential effect of the inhibitor on cancer cells and normal cells accounts for the high therapeutic index of the  
20 inhibitors of this invention when used as antineoplastic agents. Toxicity of the inhibitor to normal cells is therefore low, compared to most currently available antineoplastic agents.

## DETAILED DESCRIPTION OF THE INVENTION

### 25 I. Introduction.

All genes, except those of the two sex chromosomes, exist in duplicate in human cells, with one copy of each gene (allele) found at the same place

(locus) on each of the paired chromosomes. Each chromosome pair thus contains two alleles for any gene -- one from each parent. This redundancy of allelic gene pairs on duplicate chromosomes provides a safety system; if a single allele of any gene pair is defective or absent, the surviving allele will  
5 continue to produce the coded protein.

Because humans are genetically heterogeneous, technically referred to as DNA polymorphism, many of the paired alleles of genes differ from one another. When the two alleles are identical, the individual is said to be homozygous for that pair of alleles at that particular locus. Alternatively,  
10 when the two alleles are different, and usually the differences are small, the individual is heterozygous at that locus. Typically both alleles are transcribed and ultimately translated into proteins used by the cell.

In the homozygous situation both alleles are identical, hence proteins encoded by the two alleles are identical. In the heterozygous situation, each  
15 allele encodes a different mRNA. Depending on the effect of the nucleotide difference on the relevant codon, the mRNAs encoded by two alleles may translate into the same protein or into forms of the same protein differing by one or more amino acids. If one of a pair of heterozygous alleles is lost due to a deletion of DNA from one of the paired chromosomes, only the remaining  
20 allele will be expressed and the affected cells will be functionally homozygous. This situation is termed a "loss of heterozygosity" (LOH) or reduction to homozygosity. Following this loss of an allele from a heterozygous cell, the protein or gene product thereafter expressed will be homogeneous because all of the protein will be encoded by the single remaining allele. The cell  
25 becomes effectively homozygous at the gene locus where the deletion occurred. Almost all, if not all, cancer cells undergo LOH at some chromosomal regions.

The inventor recognized that a therapeutic strategy can be designed around the loss of heterozygosity (LOH) in cancer cells if this loss occurs in a gene expressing a protein which is necessary for cell viability or growth. This strategy begins with the identification of heterozygous alleles of genes coding for proteins essential for cell viability or growth in normal cells and the loss of one of the alleles in cancer cells. Mitelman, F., Catalog of Chromosome Aberrations in Cancer, New York: Liss (1988); Sandberg, The Chromosomes in Human Cancer and Leukemia, New York: Elsevier (1980); Seizinger, *et al.*, "Report of the committee on chromosome and gene loss in neoplasia," Cytogenet. Cell Genetics, 58:1080-1096 (1991).

Through the use of DNA probes, DNA from an individual's normal cells can be compared with DNA extracted from the same individual's tumor cells. LOH can be identified when Southern blot hybridization analysis reveals heterozygosity (dissimilar alleles) of a gene in the normal cells but homozygosity (identical alleles) of the same gene in the tumor cells. Alternatively, LOH can be assayed by demonstrating two polymorphic forms of a protein in normal heterozygous cells, and only one form in cancer cells where the deletion of an allele has occurred. A review of many published studies of LOH in cancer cells is described in Lasko, Cavenee, and Nordenskjold, "Loss of Constitutional Heterozygosity in Human Cancer," Ann. Rev. Genetics, 25:281-314 (1991).

The alternative alleles of genes coding for proteins vital for cell viability or growth are termed herein "allele A" and "allele B." Typically the loss of a single copy of any gene pair, either allele A or allele B, is not fatal. The surviving copy is able to maintain normal cell function by continuing to express the vital protein. Transformed cancer cells, in which one allele of a gene encoding a vital protein has been deleted, are similarly still capable of



producing the protein from the remaining allele. If allele B is lost due to LOH in a tumor cell, allele A still functions. However, if this tumor cell, which has only the A allelic form of the protein left, is treated with an inhibitor directed specifically to allele A, the cell will cease growth or die because either  
5 the synthesis of the vital protein or its biological activity will be suppressed by exposure to the inhibitors of this invention.

This same allele A-specific inhibitor which is fatal to the cancer cell will not kill a normal cell with two functioning alleles, A and B. The gene product produced from allele A will be inhibited in normal cells, but allele B  
10 will be unaffected and will continue to produce the vital protein, permitting normal cells to survive.

This invention is thus directed to the therapeutic use of inhibitors directed at one allele of a pair of alleles encoding a protein vital to cell growth. Various types of inhibitors can be identified which affect the function of the  
15 vital protein, or alternatively inhibit the transcription or translation of one of the alternative alleles. The result of the inhibition is a selective decrease in gene activity by specific inhibition of one allelic form of the protein.

The "inhibitors" or "antineoplastic agents" of this invention represent a new approach to cancer therapy because they are lethal only for cancer cells.  
20 The advantages of this approach include, first, lack of toxicity to the normal cells of the patient resulting in a therapeutic index greater than that of conventional cancer chemotherapy drugs, and second, it is not necessary that the inhibitors be targeted specifically to the cancer cells, as they can be administered systemically.

25 The strategy to be applied in cancer therapy is as follows: (1) the identification in a cancer patient of heterozygous loci (with alleles A and B) coding for proteins vital for cell viability and/or cell growth; (2) the identification of the loss or the absence of one of the alleles in cancer cells due

to genetic deletion and (3) the application of inhibitors with high specificity for the single remaining allele of the vital gene retained by the cancer cell after LOH.

Normal cells expressing equal amounts of allele A and allele B of the vital gene will typically show a reduction in gene activity when they take up the inhibitors of this invention, but should remain viable due to the activity of the protein encoded by the uninhibited allele. On the other hand, cancer cells expressing only one allele due to LOH, either allele A or allele B, will respond to the inhibitors of this invention which are specifically directed to the remaining allele, with a substantial reduction in gene activity. Growth of cancer cells exposed to the inhibitors of this invention will be inhibited due to the suppression of either the synthesis or the biological activity of the vital gene product. To have a high therapeutic index, an inhibitor should selectively block the activity of one allele, for example the allele A form, but have no inhibitory effect on the activity of the allele B form.

One gene can have only two allelic forms in any given individual, but a gene can have more than two alleles in the human population. According to this invention, inhibitors can be targeted to any of the alleles in the population. Thus, this invention is directed to an inhibitor with a specificity for an allele of a gene coding for a protein vital for cell viability or cell growth, where the gene has two or more alleles in the human population.

## II Identification of Alternative Alleles.

The cancer therapy strategy of this invention utilizes the genetic differences between normal cells and cancer cells. The first step in the therapeutic strategy is identifying genes which code for proteins or other factors vital to cell survival and growth that are lost through LOH in cancer

cells. Since many genes have been mapped to specific chromosomal regions, this search is best started by investigating the chromosomal regions characteristically deleted in different forms of human cancer. Table 1 from the review conducted by Lasko *et al.* in Ann. Rev. Genetics, *supra*,  
5 summarizes results of numerous studies determining loss of heterozygosity in tumors.

Once sites of deletions are identified in the chromosomes of a patient's cancer cells, genes which map to the deleted chromosomal segments and are known to code for proteins vital for cell growth or survival will be tested for  
10 DNA polymorphism. The goal is to identify all sites of LOH in the chromosomes of the cancer cells to afford the broadest selection of target genes coding for vital proteins.

TABLE 1: Loss of Heterozygosity in Human Solid Tumors

Chromosome Region	Tumor Type	Chromosome Region	Tumor Type
1p	Breast carcinoma		squamous cell carcinoma
	Cutaneous melanoma (metastatic)		basal cell carcinoma
	Medullary thyroid carcinoma:		Testicular cancer
MEN2A			Wilms tumor
	Neuroblastoma	11q	Insulinoma
	Pheochromocytoma: MEN2A		Parathyroid tumors
sporadic		12q	Gastric adenocarcinoma
1q	Breast carcinoma	13q	Adrenocortical adenoma
	Gastric adenocarcinoma		Breast carcinoma
2	Uveal melanoma		Gastric carcinoma
3p	Breast carcinoma		Hepatocellular carcinoma
	Cervical carcinoma		Lung cancer:
	Lung cancer:		small cell carcinoma
	small carcinoma		Neuroblastoma
	non-small cell carcinoma		Osteosarcoma
	large cell carcinoma		Retinoblastoma
	squamous cell carcinoma	14	Colorectal carcinoma
	adenocarcinoma	14q	Neuroblastoma
	Ovarian carcinoma	16	Breast carcinoma
	Renal cell carcinoma: familial	16q	Breast carcinoma
sporadic			Hepatocellular carcinoma
	Testicular carcinoma		Primitive neuroectodermal tumor
4q	Hepatocellular carcinoma		Prostate cancer
5q	Colorectal carcinoma	17p	Adrenocortical adenoma
	Hepatocellular carcinoma		Astrocytoma
6q	Ovarian carcinoma		Bladder carcinoma
	Primitive neuroectodermal tumor		Breast carcinoma
	Renal cell carcinoma		Colorectal carcinoma
	Testicular teratocarcinoma		Lung cancer:
9p	Glioma		small cell carcinoma
9q	Bladder carcinoma		squamous cell carcinoma
10	Glioblastoma multiforme		adenocarcinoma
10q	Hepatocellular carcinoma		Medulloblastoma
	Prostate cancer		Neurofibrosarcoma: NF1
11p	Adrenal adenoma		Osteosarcoma
	Adrenocortical carcinoma		Ovarian carcinoma
	Bladder carcinoma		Primitive neuroectodermal tumor
	Breast carcinoma		Rhabdomyosarcoma
	Embryonal rhabdomyosarcoma	17q	Breast carcinoma
	Hepatoblastoma		Neurofibroma: NF1
	Hepatocellular carcinoma	18	Renal cell carcinoma
	Lung cancer:	18q	Breast carcinoma
	squamous cell carcinoma		Colorectal carcinoma
	large cell carcinoma	22q	Acoustic neurinoma
	adenocarcinoma		Colorectal carcinoma
	Ovarian carcinoma		Ependymoma
	Pancreatic cancer		Meningioma
	Parathyroid tumors		Neurofibroma
	Pheochromocytoma		
	Skin cancer		

There are three basic criteria for selecting these genes: (1) the locus coding for the vital protein must be heterozygous, i.e., there must be two or more allelic forms in the human population; (2) the heterozygous alleles must differ in a way that permits the design of an allele-specific inhibitor,

5 which may target DNA, RNA, or protein differences; and, (3) the targeted locus must encode a protein necessary for cell growth or survival so that inhibition of the gene or its product will either reduce cell division or cell growth or kill the cell.

As used herein the term, "genes which code for proteins or factors  
10 required for cell viability" is meant to include those genes that express proteins required for cell survival as well as those genes required for cell growth in actively dividing cell populations. These genes encode proteins which can be involved in any vital cell process such as, but not limited to, DNA replication, gene transcription and translation, intracellular or  
15 transmembrane transport, regulation of transcription or translation, cellular energy metabolism, DNA repair, mitosis, signal transduction, or regulation of these functions.

One illustration is the transferrin receptor (TFRC). All actively dividing cells need iron for their growth and to sustain essential metabolic  
20 pathways. Iron is required for a large number of cellular enzymes, including cytochromes, which are necessary for oxidative phosphorylation and for ribonucleotide reductase, the enzyme catalyzing conversion of ribonucleotides to deoxyribonucleotides. The transferrin receptor participates in the cellular acquisition of iron from transferrin by receptor mediated  
25 endocytosis. The receptor is expressed on virtually all human cells. It is composed of two identical subunits held together by disulfide bonds to form a dimer of 180 kilodaltons. The gene encoding the transferrin receptor is located on chromosome 3 (q26.2-q-ter), a chromosome which frequently

undergoes loss of heterozygosity in a variety of cancers including small cell lung cancer and adult kidney cancer. All or part of chromosome 3 is deleted in virtually 100% of small cell lung cancer; in >90% of renal cancers; in ~50% of all non small cell lung cancer; in ~50% of cervical carcinomas; and in ~40% of all breast cancers. This is probably due to the fact that several tumor suppressor genes are encoded on chromosome 3.

Another illustration is human dihydrofolate reductase (DHFR). The antifolates are a class of potent anti-metabolites. Methotrexate (MTX) is the most widely used antifolate in cancer therapy. It inhibits dihydrofolate reductase, which maintains the intracellular folate pool in the form of tetrahydrofolate. The reduction of folic acid to its active form, tetrahydrofolate, occurs in two steps, in which two successive pairs of hydrogen atoms are added. The second step is catalyzed by dihydrofolate reductase. Tetrahydrofolate is an essential co-enzyme for the *de novo* synthesis of pyrimidines and purines. The reaction catalyzed by DHFR is crucial for the biosynthesis of pyrimidines and purines since there exists no redundancy in this pathway. Thus, inhibition of DHFR by methotrexate leads to cell killing.

Once a gene coding for a protein or factor vital to cell viability is identified, its genomic DNA and cDNA sequences, if not previously established, can be ascertained and sequenced according to standard techniques. Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). The next step is to test DNA from multiple individuals to determine whether the DNA sequence for the target gene is polymorphic. The target gene of this invention must occur as heterozygous alleles in the population; that is, the DNA polymorphism should either affect the primary amino acid sequence of the product of the targeted gene, which would facilitate the design

of inhibitors of the protein product, or be a mutation anywhere within the genomic DNA sequence, including the promoter or intron regions, such that it can be exploited to design inhibitors of transcription or translation which distinguish between the two heterozygous alleles of the targeted gene.

- 5 Polymorphisms that do not alter protein sequence can be targeted with antisense oligonucleotides.

The most elementary genetic variant, which is common in mammalian genomes, is the single nucleotide substitution. It has been estimated that the comparison of haploid genomes will reveal this type of  
10 variant every 300 to 500 nucleotides (Cooper, *et al.*, Human Genetics, 69:201:205 (1985)). The most common technique currently employed in the identification of such single nucleotide polymorphisms is single strand conformation polymorphism (SSCP) method. (Orita, *et al.*, "Rapid and Sensitive Detection of Point Mutations and DNA Polymorphisms Using the  
15 Polymerase Chain Reaction, Genomics, 5:874-879 (1989)). Also employed are restriction fragment length polymorphism (RFLP), heteroduplex analysis, ligase chain reaction (LCR), denaturing gradient gel electrophoresis (DGGE) (Myers, Maniatis, and Lerman, Methods Enzymol., 155:501-527 (1987)) or direct nucleotide sequencing. The SSCP method reveals the presence of  
20 sequence variation between individuals as shifts in electrophoretic mobility, but does not show the sequence itself. Direct sequencing of DNAs with altered mobility will identify the precise nucleic acid sequence differences among the various alleles. From the nucleic acid sequence data, the amino acid sequence can be determined. One example of the use of this technique is  
25 in Pelletier *et al.*, Cell, 67:437-447 (1991).

### III. Inhibitors for the Alternative Alleles.

Once two or more alleles are identified for the target vital gene, inhibitors of high specificity for the protein product of either allele A or B can be designed or identified empirically. As used herein, the term "inhibitors" is  
5 meant to include any molecule or compound capable of allele-specific blocking of transcription or translation or of allele specific inhibition of the activity performed by the vital protein.

Inhibitors that can be used in the present invention will depend on whether allelic variation at a target locus affects the amino acid sequence, the  
10 mRNA sequence, or the DNA in intron and promoter regions. If there is variation at the protein level, then classes of inhibitors to consider would include targeted low molecular weight drugs, oligopeptides and their derivatives, and antibodies, including modified or partial antibody fragments or derivatives. For mRNA or DNA polymorphism the main class of  
15 inhibitors are complementary oligonucleotides and their derivatives and catalytic RNA molecules such as ribozymes. The generation of inhibitors of this invention can be accomplished by a number of methods. The preferred method for the generation of specific inhibitors of the targeted allelic gene product relies on computer modeling of both the target protein and the  
20 specific inhibitor. Other methods include screening compound libraries or microorganism broths, empirical screening of libraries of peptides displayed on bacteriophage, and various immunological approaches. Further, in the treatment of cancer patients, a therapeutic strategy includes using more than one inhibitor of this invention to inhibit more than one target. In this  
25 manner, inhibitors directed to different proteins vital to cell growth can be targeted and inhibited simultaneously. The advantage of this approach is to increase the specificity of the inhibition of proliferation of cancer cells, while at the same time maintaining a low incidence of side effects.



### A. Targeted Drug Design.

Computer-based molecular modeling of target proteins encoded by the various alleles can be used to predict their three-dimensional structures using computer visualization techniques. On the basis of the differences between the three-dimensional structure of the alternate allelic forms of the proteins, determinants can be identified which distinguish the allelic forms. Novel low molecular weight inhibitors or oligopeptides can then be designed for selective binding to these determinants and consequent allele-specific inhibition. Descriptions of targeted drug design can be found in I. Kuntz, "Structure-Based Strategies for Drug Design and Discovery," Science, 257:1078-1082 (1992) and J. Dixon, "Computer-Aided Drug Design: Getting the Best Results," Trends in Biotechnology, 10:357-363 (1992). Specific applications of the binding of molecules to receptors using computer modeling have been described in Piper *et al.*, "Studies Aided by Molecular Graphics of Effects of Structural Modifications on the Binding of Antifolate Inhibitors to Human Dihydrofolate Reductase," Proc Am. Assoc. Cancer Res. Annual Meeting, 33:412 (1992); Hibert *et al.*, "Receptor 3D-Models and Drug Design," Therapie (Paris), 46:445-451 (1991)(serotonin receptor recognition sites). Computer programs that can be used to conduct three-dimensional molecular modeling are described in G. Klopman, "Multicase 1: A Hierarchical Computer Automated Structure Evaluation Program," Quantitative Structure-Activity Relationships, 11:176-184 (1992); Pastor *et al.*, "The Edisdar Programs Rational Drug Series Design," Quantitative Structure-Activity Relationships, 10:350-358 (1991); Bolis *et al.*, "A Machine Learning Approach to Computer-Aided Molecular Design," J. Computer Aided Molecular Design, 5:617-628 (1991); and Lawrence and Davis, "CLIX: A Search Algorithm for Finding Novel Ligands

Capable of Binding Proteins of Known Three-Dimensional Structure,"  
Proteins Structure Functional Genetics, 12:31-41 (1992).

Low molecular weight inhibitors specific for each allelic protein form can be predicted by molecular modeling and synthesized by standard organic chemistry techniques. Computer modeling can identify oligopeptides which block the activity of the product of the target gene. Techniques for producing the identified oligopeptides are well known and can proceed by organic synthesis of oligopeptides or by genetic engineering techniques. R. Silverman, The Organic Chemistry of Drug Design and Drug Action, Academic Press (1992).

The inhibitors of this invention can be identified by selecting those inhibitors that selectively inhibit the growth of cells expressing the B allelic form for example, but do not inhibit the activity of the A allelic form.

#### 15                   B.     Antibody Inhibition.

Once a vital gene is identified and is determined to exist in two or more allelic forms which encode different proteins, antibodies can be raised against both allelic forms of the protein. The techniques for using a specific protein or an oligopeptide as an antigen to elicit antibodies which specifically recognize epitopes on the peptide or protein are well known.

In one embodiment, the DNA sequence of the desired allelic form of the target gene can be cloned by insertion into an appropriate expression vector and translated into protein in a prokaryotic or eukaryotic host cell. The protein can be recovered and used as an antigen to elicit the production of specific antibodies. In another embodiment, the DNA of the desired allelic form of the target gene is amplified by PCR technology and is subsequently translated *in vitro* into protein to be used as the antigen to elicit the production of specific antibodies. A third embodiment is to use the DNA

sequence of the alternative alleles as a basis for the generation of synthetic peptides representing the amino acid sequence of the alleles for use as antigen to elicit the production of specific antibodies.

Antibodies can be generated either by standard monoclonal antibody  
5 techniques or generated through recombinant based expression systems. See generally, Abbas, Lichtman, and Pober, Cellular and Molecular Immunology, W.B. Saunders Co. (1991). The term "antibodies" is meant to include intact antibody molecules of the IgD isotype as well as antibody fragments or derivatives, such as Fab and F(ab')<sub>2</sub>, which are capable of specifically binding  
10 to antigen. The antibodies so produced will recognize only the protein produced in the allelic form which was used as an antigen to create the antibody. If the antibody was elicited by exposure to the B form of the protein, and the B form is significantly different from the A form, then the antibody will bind only the B form. If the targeted protein is expressed on the cell  
15 surface, the antibody or antibody derivative can be tested as a therapeutic. Thus, if a cancer cell has undergone LOH due to selective loss of the A allele such that it now only expresses the B form of the vital protein, treatment with anti-B antibody will kill the cancer cell, or suppress proliferation. Normal cells, which express both the A and B forms of the vital protein, will  
20 lose the activity of the B form when treated with anti-B antibody, but they will survive because they can still utilize the A form of the protein which is not recognized by the antibody.

Antibody inhibitors are most effective when they are directed against cell surface proteins or receptors. If the vital protein produced by the targeted  
25 allele is not a cell surface protein or receptor, the development of antibody inhibitors may also require a special antibody-delivery system to facilitate entry of the antibody into the tumor cells. The plasma membrane that surrounds all cells is designed to limit the entrance of most compounds.

Entry is generally restricted to small, non-charged molecules (absence of charge allows them to slip through the fatty membrane) or to those factors that can penetrate the cell using existing, specialized import mechanisms. The introduction into cells of much larger molecules, such as specific antibodies, other proteins, or peptides, will require appropriate delivery systems that are known in the art. Alternatively, the structure of the variable region of allele specific antibodies can be used as the basis for design of smaller allele specific inhibitory molecules.

10                   C    Complementary Oligonucleotides,  
                          Oligopeptides, Ribozymes, and  
                          Other Low Molecular Weight Inhibitors.

Oligopeptides can be demonstrated to have a very high degree of specificity in their interaction with functional polypeptides such as cellular enzymes, receptors or other polypeptides essential for cell viability. Methods for screening peptide sequences which have high specificity for binding to, and functional inhibition of, a specific polypeptide target have been well described previously. Scott, J.K. and Smith G.P., "Searching for Peptide Ligands with an Epitope Library," Science, 249:386-390 (1990). These methods include the screening of M13 libraries by "phage display" of polypeptide sequences as well as direct screening of peptides or mixtures of synthetic peptides for binding to or inhibition of the target functional polypeptide. The oligopeptides of this invention can be synthesized chemically or through an appropriate gene expression system. Synthetic peptides can include both naturally occurring amino acids and laboratory synthesized, modified amino acids.

Oligonucleotides or oligonucleotide analogs which interact with complementary sequences of cellular target DNA or RNA can be synthesized and used to inhibit or control gene expression at the levels of transcription or

translation. The oligonucleotides of this invention can be either oligodeoxyribonucleotides or oligoribonucleotides, or derivatives thereof, which are complementary to the allelic forms of the targeted vital gene or they can act enzymatically, such as ribozymes. Both antisense RNA and DNA  
5 can be used in this capacity as chemotherapeutic agents for inhibiting gene transcription or translation. Trojan, J., *et al.*, "Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA," Science, 259:94-97 (1993). Inhibitory complementary oligonucleotides may be used as inhibitors for cancer therapeutics because of  
10 their high specificity and lack of toxicity.

Oligopeptides and other low molecular weight inhibitors can be identified and generated by at least one of the following methods: (1) phage display to identify peptides which bind to different allelic forms; (2) direct screening of peptide mixtures using a cDNA library; (3) screening of small  
15 organic molecules present in microorganism fermentation broth for allele-specific activity; or (4) screening of compound libraries.

A specific application of generating inhibitors which are either complementary oligonucleotides or inhibitory oligopeptides is described in Holzmayer, Pestov, and Roninson, "Isolation of dominant negative mutants  
20 and inhibitory antisense RNA sequences by expression selection of random DNA fragments," Nucleic Acids Research, 20:711-717 (1992). In this study, genetic suppresser elements (GSEs) are identified by random DNA fragmentation and cloning in expression plasmids.

Preferred oligonucleotide inhibitors include oligonucleotide analogues  
25 which are resistant to degradation or hydrolysis by nucleases. These analogues include neutral, or nonionic, methylphosphonate analogues, which retain the ability to interact strongly with complementary nucleic acids. Miller and Ts'O, Anti-Cancer Drug Des., 2:11-128 (1987). Further

oligonucleotide analogues include those containing a sulfur atom in place of the 3'-oxygen in the phosphate backbone.

The references cited herein are incorporated by reference for their disclosure of published laboratory techniques. The invention is illustrated further by the following examples, which are not to be taken as limiting in any way.

#### EXAMPLE A

The steps to execute this strategy are as follows:

1. Identify by appropriate DNA based biochemical techniques the sites of additional frequent nucleic acid and/or amino acid variation in the gene vital for cell growth in the human population.
2. Express variant forms of the protein encoded by the gene vital for cell growth in a prokaryotic or eukaryotic expression system in a manner that permits selection and/or screening for cells expressing gene activity as well as selection or screening for cells in which the gene activity has been blocked by an inhibitor. This system is then used to identify inhibitors which have a high degree of specificity for one allele form of the gene over another.
3. Once these inhibitors are identified, their effectiveness in arresting (inhibiting) cell growth or causing cell death is assayed on human cells of the appropriate genotypes to test for specificity. This initial test is followed by tests of cell killing or arresting in tumor cell lines of appropriate genotypes to test relative efficacy of the inhibitors in tumor cells. Demonstration of efficacy in cell killing in cultured tumor cell lines is followed by assessment of efficacy of an inhibitor in experimental tests in animal models of human tumors such as human tumor xenografts in nude or scid mice.

4. A successful outcome of the cultured cell work in step 3 permits the initiation of clinical trials. Patients' somatic cells and tumor cells are tested to determine alleles at the target gene locus. Patients are selected for treatment if their normal cells are heterozygous for the appropriate alleles and their tumors are homozygous for the gene due to LOH at the gene locus. These patients are given systemic doses of the allele specific inhibitor. A significant reduction of tumor burden compared to untreated control animals is an indication of a successful result.

10

**EXAMPLE B****Transferrin Receptor (TFRC)**

- The basic principle which this example illustrates is the use of allele-specific antibodies against a polymorphic cell surface protein whose normal function is essential for cell growth. The gene encoding transferrin receptor is located on chromosome 3, one copy of which is frequently deleted in a variety of cancers, as described above. The activity of the transferrin receptor is essential for cell growth since it is the major route by which cells acquire iron, a vital element used by a number of cellular enzymes. Barnes, D. and Sato, G., "Methods for growth of cultured cells in serum free medium," Analytical Biochemistry 102:255-270 (1980). Direct evidence that transferrin receptor is vital for cell growth is provided by studies in which inhibition of this receptor by monoclonal antibodies leads to arrest of cell division. Trowbridge, I. and F. Lopez, "Monoclonal Antibody to transferrin receptor blocks transferrin binding and inhibits tumor cell growth in vitro," Proc. Natl. Acad. Sci. USA 79:1175-1179 (1982); Neckers, L.M. and J. Cossman, "Transferrin receptor induction in mitogen stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2," Proc Natl Acad Sci USA 80:3494-3498 (1983).

1. Scanning the TFRC gene for polymorphic differences that alter the encoded amino acid sequence in the extracellular domain (amino acids 90-760).

- 5 The TFRC cDNA sequence has previously been determined. The two published sequences of transferrin receptor are identical over the entire protein coding sequence. Schneider, Owen, Banville & Williams "Primary Structure of Human Transferrin Receptor Deduced from the mRNA Sequence," Nature 311:675-678 (1984); McClelland, Kuhn & Ruddle, "The
- 10 Human Transferrin Receptor Gene: Genomic Organization and the Complete Primary Structure of the Receptor deduced from a cDNA Sequence," Cell 39:267-274 (1994).

To investigate the possible existence of variant alleles of TFRC we screened 39 human cell lines derived from normal lymphocytes (7) and a

15 variety of tumor cell lines (32) for variation in the TFRC extracellular domain. Two standard techniques for detecting DNA sequence variation, single strand conformation polymorphism (SSCP) and heteroduplex analysis, were used to screen TFRC cDNA produced from each of the cell lines. The screening protocol consisted of 5 steps: a) isolate RNA from each of the cell

20 lines; b) produce cDNA from each of the RNA samples; c) from each cDNA sample amplify DNA encoding the extracellular domain of the TFRC in 10 separate overlapping polymerase chain reaction (PCR) products using oligonucleotide primers specific for TFRC. (See Table below summarizing details of TFRC PCR primers and products.); d) use specialized electrophoretic

25 techniques (SSCP and heteroduplex analysis) to analyze all PCR products, looking for altered electrophoretic mobility that would indicate the likely existence of sequence variants; e) sequence electrophoretic variant PCR products to determine nucleotide sequence of alternate alleles.



TFRC PCR Products

	<u>Primer Pair</u>	<u>Bases Covered*</u>	<u>Size of Fragment Produced</u>
5	T1-T2:	501-743	243 bp
	T3-T4:	689-935	247 bp
	T5-T6:	886-1136	251 bp
	T7-T8:	083-1333	251 bp
10	T9-T10:	1282-1533	252 bp
	T11-T12:	1476-1733	258 bp
	T13-T14:	1683-1920	238 bp
	T15-T16:	1870-2117	248 bp
	T17-T18:	2064-2321	258 bp
15	T19-T20:	2269-2569	301 bp

\*Numbering of bases is from Schneider, *et al.* (cited above).

Using the strategy described, we detected two DNA polymorphisms in  
 20 TFRC. The first polymorphism is at nucleotide 687 (again, numbering of  
 bases from Schneider, Owen, Banville & Williams) and was detected in the  
 243 bp product produced with primers T1 and T2 (see table above). The  
 published sequences indicate A; we found a second class of alleles with G at  
 this position. The substitution of G for A changes codon 142 of TFRC from  
 25 AGC, which encodes the amino acid serine, to GGC, which encodes glycine.  
 Thus, the two alleles encode different versions of the TFRC protein. The  
 substitution of G for A at nucleotide 687 also results in a new site for the  
 restriction endonuclease *Msp* I, which recognizes and cleaves the sequence  
 CCGG (but not CCAG). This difference in the DNA restriction pattern of the  
 30 two alleles provides the basis for a simple test for determining an  
 individual's genotype (see below). The second polymorphism is at nucleotide  
 2387 (third position of codon 708) and was detected in the 301 bp product

produced with primers T19 and T20. The published sequences indicate G at this position. We found a new class of alleles with A, which does not alter the amino acid specified by codon 708 (both ACG and ACA encode threonine). The substitution of A for G eliminates a site for the restriction endonuclease BsoF1. This difference in the DNA restriction pattern of the two alleles provides the basis for a test to determine an individual genotype.

The results of screening our sets of human samples to determine the allele frequencies at these two positions are summarized in the Tables 3 and 4 below.

10

Table 3: Genotype at position 687/codon 142

	Cell Lines*	A/Ser	G/Gly	A/G:
				Ser/Gly
15	Breast cancer	3	2	2
	Colon cancer	1	0	0
	Kidney cancer	0	0	3
	Malignant Melanoma	3	1	6
	Non-small cell lung cancer	1	3	5
20	Small cell lung cancer	1	1	0
	Lymphocytes (EBV)	2	3	2
	Totals	11	10	18

Table 4: Genotype at position 2387

25

<u>G</u>	<u>A</u>	<u>G/A</u>
24	1	8

\*Note: Tumor cell lines are not ideal for assessing allele frequencies in a population because there may be under representation of allelic diversity due to loss of heterozygosity. We used tumor cell lines only because they were readily available at the time the studies were being performed.

30

These results show that the transferrin receptor is expressed in multiple allelic forms and that the alternate alleles are widely distributed in the population. Over half of the samples analyzed were heterozygous at one or both variable nucleotide positions. Thus, human genes vital for cell growth can be polymorphic, including variation manifested at the protein level. The next step is to produce inhibitors of transferrin receptor specific for each of the allelic forms. In the case of the nucleotide 687 polymorphism, the inhibitor could be directed to DNA, RNA or protein while only DNA or RNA allele specific inhibition is possible for the nucleotide 2387 polymorphism.

10

## Materials and Methods

### Materials

Cell lines were obtained from several sources, including the American Type Culture Collection (ATCC). "ATCC Catalogue of Cell Lines & Hybridomas," 7th edition, 1992, ATCC 12301 Parklawn Drive, Rockville, MD 20852-1776. Cell lines were also obtained from The Coriell Institute for Medical Research, 401 Haddon Avenue, Camden, N.J. 08103 (described in their "1992/1993 Catalog of Cell Lines"), and from Dr. Frank Haluska (MIT Center for Cancer Research) and Dr. Vincent Stanton (formerly of MIT Center for Cancer Research, now at K.O. Technology). All enzymes were purchased from New England Biolabs, except Taq polymerase, which was purchased from Perkin Elmer. dNTPs were purchased from Pharmacia.  $\alpha^{32}\text{PdCTP}$  and  $\gamma^{32}\text{PATP}$  were purchased from New England Nuclear and MDE gel solution was purchased from AT Biochem, Malvern, PA.

25

### Methods

Cell culture: Tumor Cells were grown in DME supplemented with calf serum or fetal bovine serum. Lymphocytes were grown in RPMI 1640 supplemented with calf serum. Tissue culture was performed using standard

techniques. (I.R. Freshney, "Culture of Animal Cells: A Manual of Basic Technique," Alan R. Liss, Inc., N.Y., N.Y. (1983)).

RNA isolation: RNA was extracted by suspension of cells in guanidinium isothiocyanate followed by centrifugation through 5.7 M CsCl as described on pages 7.19-7.22 of Sambrook, Fritsch & Maniatis "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

cDNA preparation: cDNA was prepared from 1-5 µg of RNA in 13 ml of diethylpyrocarbonate (DEPC) treated water using the following protocol from Bethesda Research Labs (BRL) SuperScript kit: 1) 1 ml of random hexamer solution was added to the tube and mixed gently. 2) The solution was heat to 70°C for 10 minutes and then incubated on ice for 1 minute. 3) The following were then added: 2 µl 10X Synthesis buffer, 1 µl 10 mM dNTP mix, 2 ml 0.1 M DTT, 1 µl BRL Superscript II reverse transcriptase (200U/µl). 4) Mix gently and incubate at room temperature for 10 minutes. 5) Transfer tube to a 42°C block or water bath and incubate for 50 minutes. 6) The reaction was terminated by incubating the tube at 70°C for 15 minutes, then placed on ice. 7) 1 µl of RNase H was added to the tube and incubated at 37°C for 20 minutes.

PCR amplification of cDNA with TFRC primers: Nucleotides 501-2561 of TFRC were amplified in 10 separate PCR reactions. The primer coordinates and sizes of the overlapping PCR products are shown in the Table above. For body labelling, each PCR reaction was set up in a 10 ml volume with the following ingredients: 1 µl of cDNA, 1 µl of 10X PCR buffer (15 mM MgCl<sub>2</sub>, 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 0.1% Gelatin), 1.5 µl dNTPs (1.25 mM each: dATP, dCTP, dGTP, dTTP), 0.2 µl primer 1 (any of primers T1, T3, T5, T7, T9, T11, T13, T15, T17 or T19), 0.2 µl primer 2 (primer T2, T4, T6, T8, T10, T12, T14, T16, T18 or T20 depending on primer 1), 0.25 µl α<sup>32</sup>PdCTP (3,000

Ci/mMol), 5.7  $\mu$ l distilled water, 0.15  $\mu$ l Taq DNA Polymerase (2.5 U/ $\mu$ l). Alternatively, one PCR primer was end labelled by kinasing addition of a radioactive phosphorous to the 5' end with polynucleotide kinase; 0.25  $\mu$ l of kinased primer was substituted in place of the  $\alpha^{32}\text{P}$ dCTP in the reaction  
5 above. The kinase reaction consisted of 1  $\mu$ l oligonucleotide primer (from 20 mM stock), 1  $\mu$ l 10X Kinase buffer (as supplied by New England Biolabs), 5  $\mu$ l  $\gamma^{32}\text{P}$  ATP (3,000 Ci/mMol), 2.5  $\mu$ l distilled water and 0.5  $\mu$ l T4 Polynucleotide kinase. Amplification of templates was performed in an MJ Research PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA).  
10 Conditions for PCR were: 3 minutes 94°C, followed by 30 cycles consisting of 25 seconds at 94°C, 20 seconds at 45°C, 30 seconds at 72°C followed by 10 minutes at 72°C.

SSCP analysis: Radioactively labelled PCR products were diluted into formamide load buffer (95% formamide, 0.5 X TBE, .02% bromophenol blue,  
15 .02% xylene cyanol) and electrophoresed on 5-10% polyacrylamide gels as described by M. Orita, Y. Suzuki, T. Sekiya and K. Hayashi "Rapid and Sensitive Detection of Point Mutations and DNA Polymorphisms Using the Polymerase Chain Reaction," Genomics 5:874-879 (1989). Alternatively, radioactively labelled PCR products were analyzed on Hydrolink MDE Gels  
20 using an SSCP protocol supplied by AT Biochem (30 Spring Mill Drive, Malvern, PA 19355).

Heteroduplex analysis: Heteroduplex analysis was performed using HydroLink, a modified acrylamide manufactured by AT Biochem. Keen, J., Lester, D., Inglehearn, C., Curtis, A. and Bhattacharya, S., "Rapid Detection of  
25 Single Base Mismatches as Heteroduplexes on Hydrolink Gels," Trends in Genetics 7:5 (1991).

2. Raising antibodies which block the activity of transferrin receptor in an allele-specific manner.

Having identified TFRC variation at the protein level, we next sought to raise antisera specific for each of the two variant forms. Such antibodies would constitute allele specific inhibitors capable of arresting growth of tumor cells containing one allele, but not somatic cells from the host containing both alleles. Antibodies that distinguish the serine (Ser) and glycine (Gly) variants of TFRC could be generated by a variety of methods. We chose to first try immunizing rabbits and mice with short peptides corresponding to the Ser and Gly forms of TFRC at residue 142. (A second approach we have undertaken is described below.) We contracted with Peninsula Laboratories (Belmont, CA) to synthesize the two peptides shown below. We then coupled these peptides to Bovine Serum Albumin (BSA) or Keyhole limpet hemocyanin (KLH) and immunized rabbits. See generally Harlow, E. and D. Lane, "Antibodies," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988); Coligan, J.E., et al. "Current Protocols in Immunology," Wiley Interscience, N.Y. (1990). Six rabbits and mice were immunized with the Ser peptide:

NH<sub>2</sub>-CYS-NLE-LYS-LEU-ASP-SER-THR-ASP-PHE-THR-SER-THR-ILE-LYS-LEU-LEU-ASN-GLU-CYS-COOH

and four rabbits were immunized with the Gly peptide:

NH<sub>2</sub>-CYS-NLE-LYS-LEU-ASP-SER-THR-ASP-PHE-THR-GLY-THR-ILE-LYS-LEU-LEU-ASN-GLU-CYS-COOH.

Each of these peptides contains 3 residues not found in TFRC: Cys residues at either end to facilitate coupling of the peptides to BSA or KLH via the cross-linker sulfo-succinimidyl 4-(maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC), and a non-natural norleucine residue (nle) just after the -NH<sub>2</sub> terminal Cys to allow easy assessment of the success of coupling by determination of the nle content of the coupled material. Amino acid analysis of the coupled material (performed by Protein Structure Facility, University of Michigan Medical School, Ann Arbor, MI) showed approximately 4 peptides were coupled to each BSA molecule. Mouse immunization schedule was as follows: immunize Balb/c mice with ~15µg of peptide-BSA in 0.5 ml Freund's adjuvant on day 0 (complete adjuvant), 16 (incomplete adjuvant on day 16 and thereafter), 41, 62 and 86, bleed on days 0, 50, 75, and 89 (terminal bleed) and harvest spleens on day 89 for possible fusion to mouse lymphoma cell lines (SP2 or NS1). We contracted with Pel-Freez Biologicals (Rogers, AR) to immunize rabbits according to the following schedule, using 1 mg of antigen in 2 ml of 50% Freund's adjuvant for each immunization:

	<u>Day</u>	<u>Bleeding and Immunization Schedule</u>	
20	0	PreBleed	Immunize
	14		Immunize
	28		Immunize
	35	Bleed	
	42	Bleed	Immunize
25	49	Bleed	
	56	Bleed	Immunize
	63	Bleed	
	70	Bleed	
	77	Bleed	
30	84	Terminal Bleed	

Sera from mice and rabbits are tested for immune reactivity specific to either the Ser or Gly form of transferrin receptor. Initially, specific reactivity against the immunizing peptides is evaluated. Two 16 residue peptides identical in sequence to those described above, but without the two terminal  
5 Cys residues or the nle residue, were synthesized by SynPep (Dublin, CA) and coupled to ovalbumin with glutaraldehyde (see Harlow and Lane, *supra*). By shortening the peptide, using a different crosslinker, and coupling the antigen to ovalbumin instead of BSA, it is possible to measure only antibodies raised against the relevant segment of the immunizing peptide. Next, the immune  
10 sera is evaluated for recognition and binding to immunizing peptide in the presence of blocking peptide (the other allelic form of the receptor). That is, whether sera from a rabbit immunized with Ser peptide can recognize Ser peptide in the presence of an excess of Gly peptide, or whether sera from a rabbit or mouse immunized with Gly peptide can recognize Gly peptide in the  
15 presence of an excess of Ser peptide. The allele specific antisera identified by this test are next tested for the same reactivity against the whole transferrin receptor. (Some antisera raised against peptide antigens fail to detect the immunizing peptide when it is part of a larger protein.) If allele specific antibodies are detected against one or both forms of the transferrin receptor,  
20 the antibodies are purified by affinity to the immunizing peptide bound to a column in the presence of soluble blocking peptide (i.e., the non-immunizing peptide, which will bind and remove antibodies reactive against both forms of TFRC).

Spleens harvested from the immunized mice or rabbits are used to  
25 generate monoclonal antibodies using the recombinant phage antibody system sold by Pharmacia Biotech. McCafferty, *et al.*, Nature, 348:552 (1990). Finally, tumor cells expressing Allele A of the transferrin receptor are incubated with antibodies specific for Allele A and tumor cells specific for



Allele B are incubated with antibodies specific for Allele B. In both cases, the tumor cells cease proliferating or die due to the antibodies binding to the specific allele, which interferes with the transferrin receptor function. Heterozygous tumor cells incubated with either of the two allele specific  
5 antibodies do not die.

To generate blocking antibodies, the antibodies are chimerized. Therefore, the light chain variable region of the corresponding antibody is fused to the human C $\kappa$  region and the heavy chain variable region is fused to the human IgD constant region since this region has no effector functions  
10 such as complement fixation or binding to Fc-receptors. The chimeric gene constructs for the light and heavy chain are transfected into myeloma cells such as PS/20 (see Coligan *et al.*, *supra*). This cell line does not synthesize endogenous heavy and light immunoglobulin chains. Chimeric TFRC allele specific monoclonal antibodies are isolated from culture supernatants and  
15 after purification are tested in nude mice carrying human tumors which are homozygous for the targeted allele so that it can be ascertained that there are no barriers impeding access of the antibody to the extracellular domain of the TFRC in the membrane of the tumor cells *in vivo*.

Strategies to identify allele specific inhibitors at the DNA or RNA  
20 levels for either the nucleotide 687 or 2387 polymorphisms are based on previously described approaches to antisense therapeutics as described, for example, in Toulme and Helene, "Antimessenger oligodeoxyribonucleotides; an alternative to antisense RNA for artificial regulation of gene expression -- a review," Gene, 72:51-58 (1988) and Inouye, Antisense RNA; its functions  
25 and applications in gene regulation -- a review," Gene, 72:25-34 (1988). For an account of successful allele specific antisense suppression of a mutant Harvey ras gene see, Monia, *et al.*, "Selective Inhibition of Mutant Ha-ras mRNA Expression by Antisense Oligonucleotides," J. Biol. Chem., 267: 19954 (1992).

For a description of ribozyme based strategy see, Gerlach and Young in Chapter 8, "Ribozymes for the Control of Gene Activity *in vivo*," from Antisense Nucleic Acids and Proteins: Fundamentals and Applications, edited by J.N.M. Mol & A. R. van der Drol. Marcel Dekker, Inc. NY (1990);

- 5 Cotten and Birnstiel, "Ribozyme mediated destruction of RNA *in vivo*," EMBO L. 8:3861-3866 (1989); and Cameron and Jennings, "Specific gene suppression by engineered ribozymes in monkey cells," PNAS USA, 86:9139-9143 (1989).

10

3. Alternate strategy for generation of allele specific anti-transferrin antibodies.

- A second approach we are undertaking is to generate allele specific antibodies in mice using a different strategy. This approach requires making
- 15 two mouse strains, each transgenic for one of the two allelic forms of human transferrin receptor (serine or glycine at residue 142). Transgenic mice which have integrated the serine or glycine allele of human TFRC in their genome will be identified by Southern blot analysis using human TFRC receptor specific DNA probes and by PCR analysis using TFRC specific oligonucleotide
- 20 primers. Expression of human TR receptor on the cell surface of lymphocytes will be tested by indirect immune staining using monoclonal human transferrin specific antibodies as first antibodies and Fluorescein labeled anti murine antibodies as second antibodies. After confirmation of the expression of the two allelic forms of human TR on the surface of murine lymphocytes,
- 25 we will immunize the transgenic mice expressing the serine 142 allelic form of human TR with lymphocytes derived from transgenic mice expressing the glycine allele of human TR. The only difference between the two transgenic mice is the one amino acid difference in the human TR. The immunization of these mice should therefore elicit an immune response against the glycine
- 30 epitope of human TR. In order to obtain TFRC serine specific antibodies we

will immunize transgenic mice expressing the amino acid 142 glycine allelic form of human TR with lymphocytes derived from serine 142 transgenic mice. After repeated immunization of the two transgenic mice with the two immunogens, we will generate monoclonal antibodies by hybridoma  
5 technology or the phage display method. The phage display method will be used to clone those variable region genes which show the highest affinity to lymphocytes derived from the two transgenic animals. In a later step, the light chain variable region genes will be fused to C<sub>k</sub> and the heavy chain variable region gene to the immunoglobulin delta constant region.  
10 Alternatively, we can express them as antibody fragments without the constant region domains.

#### Methods

Transgenic mice will be made by microinjecting Ser or Gly specific  
15 cDNA expression constructs into mouse eggs of the same inbred mouse strain. For higher expression in the transgenic mice, an intron DNA sequence can be added. For B cell specific expression, we can use an immunoglobulin promoter or, for T cell specific-expression, we can use the q or theta promoter, for example. Alternatively, we can use the CMV promoter  
20 as a ubiquitously expressing promoter.

#### 4. Efficacy Testing on Human Cells.

The identified allele-specific inhibitors must be tested for their ability to inhibit transferrin receptor activity in normal human cell lines of  
25 appropriate genotype such as fibroblasts or EBV transformed lymphoblastoid cell lines.

The allele specific antibodies or antisense constructs or other inhibitors will be tested for their ability to inhibit transferrin receptor activity in well

known tumor cell lines of the appropriate genotype, especially those known to undergo frequent LOH at the transferrin receptor locus, such as small cell lung cancer, renal cancer, non small cell lung cancer, cervical carcinomas, and breast cancer. The genotype of these tumor cell lines at the TFRC locus is  
5 determined. To genotype the tumor cells, RNA or genomic DNA is extracted, the relevant region of the gene is amplified by PCR from cDNA or DNA assayed by an RFLP technique (MspI or BsoFI digestion), by SSCP, by oligonucleotide hybridization, or by direct sequencing to determine the genotype. Those which are homozygous for the appropriate allele are  
10 screened for transferrin receptor inactivation and consequent arrest of cell division by the various allele-specific inhibitors.

#### 5. Human Clinical Trials.

Patients with tumors known to delete one copy of chromosome 3  
15 (resulting in LOH at the transferrin receptor locus) are candidates for treatment with the antibody inhibitors of this invention. Patients appropriate for treatment with a specific inhibitor can be identified by a two step genetic screening test requiring a blood sample and, for constitutionally heterozygous patients, a small sample of the patient's tumor. The first part of the test  
20 requires only blood (or any other source of nonmalignant somatic cells). The patient's allelic status (genotype) at nucleotides 687 and 2387 of TFRC is determined by PCR amplification of DNA segments surrounding each of the polymorphic sites followed by allele specific oligonucleotide hybridization or restriction endonuclease digestion with MspI (to determine genotype at 687)  
25 or BsoFI (to determine genotype at 2387). A tumor biopsy is obtained only from patients heterozygous at position 687 or 2387 or both. The method for determination of the tumor genotype depends on the amount of tumor DNA available. If  $\geq 5$  mg is available the DNA will be digested with MspI or BsoFI

as appropriate, electrophoresed on an agarose or polyacrylamide gel, blotted and hybridized with the suitable TFRC fragment. Southern blotting allows absolute quantitation of allele dosage. If < 5 mg of tumor is available the genotype is assayed by a PCR based method such as SSCP which is adapted for  
5 quantitative studies. Once patients with appropriate genotypes have been identified the responsiveness of their tumor cells to the targeted inhibitors is assayed in tissue culture. First, cell cultures of the tumor and of normal lymphocytes can be established. These cultures are then treated (screened) with the appropriate allele-specific inhibitors of transferrin receptor. A  
10 therapeutically effective inhibitor is one which kills the tumor cell line, while permitting growth of the lymphocyte-derived line (the patient's normal cells), albeit possibly slower growth. An allele-specific antibody which differentiates between tumor and normal cells is then administered therapeutically to the patient.

15

### EXAMPLE C

#### Dihydrofolate Reductase (DHFR)

As described above the DHFR gene encodes a protein essential for cell  
20 proliferation. Inhibitors of DHFR, particularly, methotrexate (MTX), are widely used in cancer chemotherapy. currently the most widely used antifolate in cancer therapy.

The DHFR gene has been cloned and its cDNA sequence has been determined several times. (Morandi *et al.*, "Multiple Forms of Human  
25 Dihydrofolate Reductase Messenger RNA," J. Mol. Bio., 156:583-607 (1982); Masters and Attardi, The nucleotide sequence of the cDNA coding for the human dihydrofolic acid reductase," Gene, 21:59-63 (1983); Masters *et al.*, "A Human Dihydrofolate Reductase Pseudogene and Its Relationship to the

Multiple Forms of Specific Messenger RNA," J. Mol. Bio., 167:23-36 (1983)). The gene is located on chromosome 5q11.2-q13.2, a region frequently reduced to homozygosity in colorectal cancer and in liver cancer. We subdivided the cDNA sequence, which comprises 979 bp into 5 overlapping fragments. The

5 fragments were generated by PCR using 10 specific oligonucleotides (D1-D10) and the same cDNAs used for the human transferrin receptor study described above. The PCR fragments, between 219 bp and 263 bp in length, were analyzed by SSCP analysis as described in Example B. The oligonucleotides used were:

10

D1: 5'-GAGGTCCTCCCGCTGCTGTC-3'  
D2: 5'-CTTGAGTTCTCTGCTGAGAAC-3'  
D3: 5'-CATTCCTGAGAAGAATCGACC-3'  
D4: 5'-GTCTTGCATGATCCTTGTCAC-3'  
15 D5: 5'-GAAGCCATGAATCACCCAG-3'  
D6: 5'-CTTAAACTAGAAAACACCTTC-3'  
D7: 5'-GTACAAATTTGAAGTATATG-3'  
D8: 5'-GGTCTTGGAAGAAGCACTTAG-3'  
D9: 5'-CATTTATGAGACATTCTTGC-3'  
20 D10: 5'-GCTTTTGGTATTTCCACTAG-3'

The radiolabelled PCR products were analyzed on a nondenaturing acrylamide gel +/- 10% glycerol. We identified two DNA polymorphisms, at nucleotide 721 and 829 (numbering of nucleotides from Genbank, accession

25 number J00140). Three of 22 tested cDNAs were heterozygous for T or C at nucleotide 829. The other 19 cDNAs were homozygous for C. At nucleotide 721, four of 20 cDNAs were heterozygous for A or T. 16 other cDNAs were homozygous for T. These nucleotide substitutions, which do not result in an amino acid exchange, are ideal targets to develop antisense oligonucleotides

or ribozymes which will specifically discriminate between the different polymorphisms.

- 5        Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one skilled in the art that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An inhibitor with a specificity for an allele of a gene coding for a protein vital for cell viability or cell growth wherein said gene has two or  
5 more alternative alleles and wherein said inhibitor blocks only one of the alternative alleles.
2. An inhibitor with a specificity for an allele of the transferrin receptor gene wherein said gene has two or more alternative alleles and  
10 wherein said inhibitor blocks only one of the alternative alleles or blocks protein expressed from only one of the alternative alleles.
3. The inhibitor of claim 2 wherein said TRFC gene has at least two alternative alleles, wherein one allele has a G instead of an A at the first  
15 position of codon 142 (nucleotide 687).
4. The inhibitor of claim 2 wherein said TRFC gene has at least two alternative alleles, wherein one allele has an A instead of a G at the third  
20 position of codon 708 (nucleotide 2387).
5. An inhibitor with a specificity for an allele of the human dihydrofolate reductase (DHFR) gene wherein said gene has two or more alternative alleles and wherein said inhibitor blocks only one of the  
25 alternative alleles or blocks protein expressed from only one of the alternative alleles.



6. The inhibitor of claim 5 said DHFR gene has at least two alternative alleles, wherein one allele has an A instead of a T at nucleotide 721.

5 7. The inhibitor of claim 5 said DHFR gene has at least two alternative alleles, wherein one allele has a T instead of a C at nucleotide 829.

8. The inhibitor of any of claims 1-7 wherein said inhibitor is an antibody or antibody fragment or derivative thereof; an oligopeptide or a derivative thereof; a complementary oligonucleotide or a derivative thereof;  
10 a complementary oligodeoxyribonucleotide or oligoribonucleotide or derivatives thereof; a ribozyme; a targeted drug; or a low molecular weight compound.

15 9. A method for producing an inhibitor against only one alternative allele of a gene having two alternative alleles comprising:

(a) identifying a target gene vital to cell viability or cell growth that has alternative allelic forms in a noncancerous cell and wherein one of said alternative allelic forms is deleted in a cancer cell and

20 (b) generating an inhibitor against the alternative allelic form present in said cancer cell.

10. A method for treating a patient suffering from a form of cancer comprising:

25 (a) screening normal cells and cancer cells from a patient suffering from a cancer to identify loss of heterozygosity in a gene vital to cell viability or cell growth in said cancer cells; and,

(b) for those patients with a loss of heterozygosity and with alternative allelic forms of said gene vital to cell viability or cell growth, administering an inhibitor with a specificity for the alternative allele present in said cancer cell.

5

11. The method of claim 10 wherein in step (a) said normal cells and said cancer cells are first cultured and then screened with at least one allele-specific inhibitor.

10

12. The method of claim 10 wherein in step (a) said normal cells and said cancer cells are screened through hybridization of DNA extracted from those cells with at least one allele-specific probe, restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, single strand conformation polymorphism (SSCP),

15 ligase chain reaction (LCR), or direct nucleotide sequencing.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/08473

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF AACR, VOL. 26, ISSUED MARCH 1985, J.K. SATO ET AL., "MONOCLONAL ANTIBODIES AGAINST DIHYDROFOLATE REDUCTASE FROM METHOTREXATE-RESISTANT L1210 CELLS", PAGE 229, ABSTRACT NO. 903, ENTIRE ABSTRACT.	1-12
Y	PROCEEDINGS OF AACR, VOL. 27, ISSUED MARCH 1986, J.K. SATO ET AL., "METHOTREXATE (MTX) RESISTANCE IN HUMAN OSTEOSARCOMA CELLS AS DETECTED BY MONOCLONAL ANTIBODIES RAISED AGAINST L-1210/R-6 DIHYDROFOLATE REDUCTASE", PAGE 258, ABSTRACT 1023, ENTIRE ABSTRACT.	1-12

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	g	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

28 OCTOBER 1994

Date of mailing of the international search report

NOV 09 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CHRIS EISENSCHENK

Telephone No. (703)308-0196

## INTERNATIONAL SEARCH REPORT

Int. national application No.

PCT/US94/08473

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER RESEARCH, VOL. 46, ISSUED APRIL 1986, R. TAETLE ET AL., "MECHANISMS OF GROWTH INHIBITION BY ANTI-TRANSFERRIN RECEPTOR MONOCLONAL ANTIBODIES", PAGES 1759-1763, ENTIRE DOCUMENT.	1-12
Y	PROC. NATL. ACAD. SCI. USA, ISSUED FEBRUARY 1982, I.S. TROWBRIDGE ET AL., "MONOCLONAL ANTIBODIES TO TRANSFERRIN RECEPTOR BLOCKS TRANSFERRIN BINDING AND INHIBITS TUMOR CELL GROWTH <i>IN VITRO</i> ", PAGES 1175-1179, ENTIRE DOCUMENT.	1-12

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/08473

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07K 16/40, 16/30, 16/28; A61K 39/00, 121/00, 123/00

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/388.26, 388.22, 388.8, 388.85; 424/143.1, 146.1, 155.1

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

530/388.26, 388.22, 388.8, 388.85; 424/143.1, 146.1, 155.1

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, EMBASE, BIOSYS, LIFESCI

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